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http://dx.doi.org/10.1289/ehp.1205739

Online 10 May 2013

NIH National Institute of Environmental Health Sciences

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Running title: Relative Effect Potencies of Dioxin-Like Compounds

Key Words: dioxin-like polychlorinated biphenyl (DL-PCB); free thyroxin (FT4); polychlorinated dibenzo-p-dioxins (PCDDs); polychlorinated dibenzo-p-furans (PCDFs); relative Effect Potency (REP); thyroid volume; toxic equivalency factor (TEF).

Acknowledgements: This work was supported by the EU 5th Framework Programme project "Evaluating human health risk from low-dose and long-term PCB exposure" QLK4-CT-2000-00488 (PCBRISK), EU 7th Framework Programme project No: 226694 "The development, validation and implementation of human systemic Toxic Equivalencies (TEQs) as biomarkers for dioxin like compounds" (SYSTEQ), by the Competence Center for SMART Technologies for Electronics and Informatics Systems and Services, ITMS 26240220072, funded by the Research & Development Operational Programme from the ERDF and Scientific Grant Agency VEGA (Bratislava, Slovakia) grant No. 1/0120/12. This research also received support from the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences.

We would like to acknowledge Lesa L. Aylward, Ph.D. Principal at Summit Toxicology Falls Church, VA 22044 for suggestions and ideas she gave us in the initial stages of our research.

Competing Financial Interests: None

Abbreviations:

AhR: Arylhydrocabon receptor

BMC: Benchmark concentration

BMCL: Benchmark concentration lower confidence limit

BMR: Benchmark response

DLCs: Dioxin-like compounds

DL-PCBs: Dioxin-like polychlorinated biphenyls

FT4: Free thyroxin

HpCDD: Heptachlorodibenzo-p-dioxin

HpCDF: Heptachlorodibenzofuran

HxCDD: Hexachlorodibenzo-p-dioxin

HxCDF: Hexachlorodibenzofuran

LOD: Limit of detection

NDL-PCBs: Non dioxin-like polychlorinated biphenyls

OCDD: Octachlorodibenzo-p-dioxin

OCDF: Octachlorodibenzofuran

PCBs: Polychlorinated biphenyls

PCDDs: Polychlorinated dibenzo-p-dioxins

PCDFs: Polychlorinated dibenzofurans

r: correlation coefficient

REPs: Relative Effect Potencies

RPF: Relative Potency Factor

SPE: Solid phase extraction

TSH: Thyroid-stimulating hormone

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

WHO TEFs: World Health Organization toxic equivalency factors

WHO TEQs: World Health Organization toxicity equivalents

Abstract

BACKGROUND: Toxic Equivalency Factors (TEFs) are an important component in the risk assessment of dioxin-like human exposures. At present, this concept is mainly based on *in vivo* animal experiments with oral dosage. Consequently, the present human TEFs derived from mammalian experiments are only applicable for exposure situations in which oral ingestion occurs. Nevertheless, these ”intake” TEFs are commonly, but incorrectly used by regulatory authorities to calculate “systemic” Toxic Equivalents (TEQs) based on human blood and tissue concentrations and consequently considered to be biomarkers for either exposure or effect.

OBJECTIVES: To determine relative effect potencies (REPs) for systemic human concentrations of dioxin-like mixture components using thyroid volume or FT4 serum concentration as the outcomes of interest.

METHODS: We compared the strength of association between each dioxin-like compound and thyroid endpoints in 320 adults residing in an area of eastern Slovakia polluted by organochlorines using a benchmark concentration and regression-based approach.

RESULTS: REPs calculated from thyroid volume and FT4 were similar. The regression slope derived REP data from thyroid volume and FT4 level correlated with the WHO TEF values (Spearman r=0.69, p=0.01 and r=0.62, p=0.03, respectively). The calculated REPs were mostly within the minimum and maximum values for *in vivo* REPs derived by other investigators.

CONCLUSIONS: Our REPs calculated from thyroid endpoints realistically reflect human exposure scenarios as they are based on chronic, low dose human exposures and on biomarkers reflecting body burden. Compared to previous results they suggest higher sensitivity to the effects of dioxin-like compounds.

Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs, dioxins), polychlorinated dibenzofurans (PCDFs, furans) and polychlorinated biphenyls (PCBs) are ubiquitous environmental compounds. PCDDs and PCDFs are combustion or industrial byproducts with no commercial use, while PCBs were frequently used in a variety of commercial applications, such as coolants and lubricants in transformers, capacitors, and other electrical equipment. Some PCBs act in a mechanistic way similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and are usually referred to as dioxin-like PCBs (DL-PCBs). The three groups of compounds are commonly found in mixtures in the environment and human food chain, usually containing a large number of congeners, such that each mixture has its own degree of dioxin-like toxicity. For risk assessment purposes, these individual compounds were assigned a toxic equivalency factor (TEF) value relative to the toxicity of TCDD by the World Health Organization (van den Berg et al. 2006). This factor indicates a relative toxicity compared to the most toxic congener, TCDD, which is given a reference value of 1. Prerequisites for this TEF concept are the exclusive inclusion of toxic effects that are mediated via the arylhydrocabon receptor (AhR) and an additive mechanism of action for mixtures of these compounds. Otherwise mediated toxic effects of PCDDs, PCDFs and PCBs cannot be quantified for risk assessment by this method.

The re-evaluation of TEF values for these compounds has become a continuous process based on available results from *in vivo* as well as *in vitro* studies. Although many studies with human cell-lines or primary cells have been published to date (Haws et al. 2006), human *in vivo* data that may contribute to the TEF concept have not been published until now. In an attempt to fill this gap, we examined cross-sectional data on thyroid impairment in a population exposed to a

mixture of organochlorines to identify relationships between individual mixture components and thyroid volume and free thyroxin (FT4). Based on these results, we estimated the relative potencies (REPs) of PCDD, PCDF and DL-PCB congeners in adult humans.

Materials and Methods

Participants. Our initial sample of 2047 adults was drawn from a population living in the towns and villages of the Michalovce, Svidnik, and Stropkov districts in eastern Slovakia, an area known to be contaminated by a mixture of organochlorines (Jursa et al. 2006; Langer et al. 2007c; Petrik et al. 2006). Adult participants were recruited between August 2001 and February 2002 with the help of primary care physicians by random selection from alphabetical lists of their patients; nearly all those approached agreed to participate. We have complied with all applicable requirements of the U.S.A. and/or international and national regulations (including IRB approval). All human participants gave written informed consent prior to the study.

Although we did not collect data on place of birth, we assumed that all subjects spent the majority of their adult life residing in these districts in agreement with low labor mobility in Slovakia. Individuals having a mild chronic controlled illness (e.g. rheumatism, hypertension, diabetes, thyroid disorders, non-morbid obesity, allergy, etc.) were not excluded from the study. At enrollment, participants were given a physical examination by our field medical staff, and sociodemographic and medical questionnaires were completed (Langer 2010; Langer et al. 2006; Langer et al. 2007a, 2007b, 2007c; Langer et al. 2008; Rádiková et al. 2008; Ukropec et al. 2010).

Whole blood samples were also collected from fasting subjects into anticoagulant-free Vacutainer™ tubes (S-Monovette, Sarstedt, Nürnberg, Germany) and centrifuged after clotting at 3000 rpm for 15 minutes. The serum was frozen at –18° C in glass vials and stored. The study protocol was approved by the institutional review board at the Slovak Medical University in Bratislava.

Chemical analyses. Of the 2047 adults selected, 320 were willing to provide 90 ml of blood for analysis of PCDDs, PCDFs, and PCBs. The serum samples were treated using a modified version of the method by Turner et al. (1994). Each thawed serum sample (5 – 30 ml) was spiked with $^{13}C_{12}$-labelled standards (fifteen 2,3,7,8-substituted PCDDs/PCDFs, twelve DL-PCBs and eleven $^{13}C_{12}$-labelled NDL-PCBs (Cambridge Isotope Laboratories Inc., Andover, MA, USA; Wellington Laboratories Inc., Ontario, Canada) 24 hours before sample processing. After the serum had been treated with diluted formic acid, the analytes were isolated by solid phase exctraction procedure using 10-g C18-column (UCT Inc., Bristol, PA, USA). A hexane extract was cleaned-up on a Power-Prep™ semi-automated cleanup system (FMS Inc., Waltham, MA, USA) with pre-packed disposable silica, alumina and carbon columns. A combined dichloromethane/n-hexane (2:98, v/v) and dichloromethane/n-hexane (50:50, v/v) eluate fractions contained mono-ortho and NDL-PCBs. A toluene eluate fraction contained PCDDs/Fs and non-ortho PCBs. The eluate fractions were concentrated and then diluted with $^{13}C_{12}$-labelled recovery standards. An HP 6890 Plus GC (Hewlett-Packard, Palo Alto, CA, USA) coupled to an MAT 95XL (Thermo Finnigan, Bremen, Germany) operating at a 10-% valley resolution of 10000 in the selected ion monitoring mode was used for 2,3,7,8-substituted PCDDs/PCDFs and PCBs determination. PCDD/F and non-ortho-PCB congeners were separated on a 30 m × 0.25 mm × 0.25 μm DB-5ms capillary column (J&W Scientific, Folsom, CA, USA). Mono-ortho and

NDL-PCB congeners were separated on a 60 m × 0.25 mm × 0.25 µm DB-5ms capillary column (J&W Scientific, Folsom, CA, USA). An analysis batch consisted of 14 serum samples, one solvent blank, and one spiked porcine serum in-house reference material. A human serum certified reference material was analyzed in each 3rd batch (Kocan et al. 2004). The qualitative and quantitative analyses were carried out using the US EPA 1613 and 1668 (US Environmental Protection Agency, 1994 and 1999) isotope dilution methods. Two congeners (1,2,3,7,8,9-HxCDF and PCB-77) were not included in the statistical analysis. The concentrations of 1,2,3,7,8,9-HxCDF congener were below the limit of detection (0.22 – 3.1 pg/g lipid) in all analyzed samples, and PCB-77 was not quantified due to the presence of high background levels in laboratory blanks.

All analytical measurements were carried out at the National Reference Centre for Dioxins and Related Compounds (Department of Toxic Organic Pollutants, Slovak Medical University) which has been certified by Slovak National Accreditation Service (ISO/IEC 17 025:2005, Certif. No. S 111, http://www.snas.sk/index.php?p=4&l=en&ts=1&id_druh=1) and regularly participates in interlaboratory studies and proficiency tests on dioxins and PCBs in food and feed. An analysis batch consisted of 14 serum samples, method blank and QC sample (porcine serum spiked with native PCDD, PCDF and PCB congeners as an in-house reference material). The certified human serum (SRM 1589a, NIST) was analyzed in each 3rd batch. Control charts were plotted for QC samples, blanks and verification calibration standards as a basis for check of accuracy, precision and reliability of the analytical process.

An enzymatic method based on the determination of total cholesterol, free cholesterol, phospholipids and triglycerides was used to determine total lipids in all the serum samples

analyzed (Akins et al. 1989), and the values were used to present the organochlorine concentrations on a lipid weight basis.

Assessment of thyroid outcomes. Thyroid examination was performed with a portable ultrasound diagnostic device (Siemens SonolineSI-400, North Rhine-Westphalia, Germany) using a 7.5 MHz linear transducer. The thyroid volume (ml) for each lobe was calculated according to the ellipsoid formula: width (cm) * length (cm) * thickness (cm) * a correction factor of 0.479 (Brunn et al. 1981). The measurements were made on each subject lying supine with the neck hyperextended and were performed by the same physician with long-term experience in field surveys and clinical ultrasound diagnostics. The physician was unaware of toxicant concentrations among participants. The intra-observer variation estimated (Ozgen et al. 1999) by 3 subsequent measurements of 50 thyroid volumes ranging between 3.0 and 20.5 ml (median 6.2 ml) was 3.9±3.5 % (mean ± SD).

FT4 was determined from stored specimens with an electrochemiluminiscent immunoassay using the automatic Elecsys system (Roche, Basel, Switzerland), as discussed previously (Langer et al. 2005).

Statistical analysis. Two approaches were used for estimation of the relative potencies of individual components of the mixture. The first, suggested previously (Fattore et al. 2004; Yang et al. 2010), was based on a comparison of benchmark concentrations (BMCs) calculated for thyroid outcomes as a function of organochlorine serum concentrations. The other was based on comparing the magnitude of the regression coefficient (β) for thyroid volume or FT4 serum concentration regressed on serum concentration of the individual congeners, analogous to Brown and colleagues (2001). We considered participant's sex and age at blood draw as potential

confounders, as well as PCDDs, PCDFs and PCBs determined in the exposure mixture. Thus multivariable regression was used when calculating REPs with adjustment for sex, age and presence of other organochlorines. For confirmation of TCDD as an index (reference) congener (US EPA 2000), we used multiple regression with backwards elimination (variable removal at $p>0.1$). The REPs resulting from both approaches were compared with published data on REPs for DLCs (Haws et al. 2006) and with the recent WHO-TEF values (van den Berg et al. 2006).

Estimation of REPs through comparison of BMCs. For each individual PCDD, PCDF and DL-PCB congener, we calculated the BMC (Crump 1995) for thyroid volume and FT4 serum concentration endpoints, using software developed by one of the co-authors (Dedík 2012). Sex and age were adjusted for in all statistical models. The BMCs for changes in thyroid volume and serum FT4 associated with TCDD concentration were compared with the BMCs of individual congeners and used to derive the congener specific REPs. Thus BMC_{TCDD}/BMC_i is the relative potency REP_i, for the i-th congener, relative to TCDD.

Estimation of REPs through comparison of regression coefficients. We calculated the regression coefficient (β) for each congener from all concentration data >LOD. We considered sex, age, PCDD, PCDF and PCB congeners identified in the mixture as confounding variables. We calculated the BMCs for most probable combinations of confounders, but listed (Supplemental Material, Table S1) a reasonable number of those which had the greatest influence on benchmark concentrations. As the addition of other organochlorines had negligible influence on model data, results are presented with adjustment for age and sex only. The REPs of the individual congeners were calculated as the ratio of the slope (β) obtained for the i-th congener to the slope (β) for TCDD, as β_i/β_{TCDD}.

Results

Participant characteristics. The subgroup of 320 subjects with complete data consisted of 203 males (mean±SD age 44.9±11.47, median 48 years) and 127 females (mean±SD age 47.3± 9.24, median 48 years), with an overall mean age of 45.8±10.7 years (median 48 years). Among males, age ranged from 20 to 75 years and in females, from 21 to 70 years. The median and mean serum concentrations, in pg WHO TEQ/g lipid of DLCs in these subjects are shown in Supplemental Material, Table S2. The data on mean and median serum concentration of PCDD, PCDF, DL-PCB congeners and the most abundant NDL-PCB congeners, of samples with concentration >LOD, are shown in Supplemental Material, Table S3. The median concentrations (Supplemental Material Table S3) of individual congeners >LOD correlated with median concentrations overlapping with TCDD >LOD (r=0.998). It can be concluded that parameters calculated from samples overlapping with TCDD >LOD represent well those from samples >LOD.

For males and females the mean±SD and median of volume of the thyroid gland were 11.56±4.42, 10.20 and 9.49±4.75, 8.35 ml, respectively. Mean±SD and median serum concentrations of FT4 for males and females were 16.93±2.65, 16.7 and 15.72±3.22, 15.39 pmol/l, respectively.

Identification of the index congener. There is general agreement that the index compound, TCDD, is the most well studied member of its class, and provides the largest body of acceptable scientific data (US EPA 2000). At the same time, the index chemical should be potent with regard to the expected endpoint. Multiple regression with backwards elimination was used to query the selection of TCDD as the index congener in concurrence with other PCDD or PCDF

congeners. We created four models (A-D) for this purpose. When we entered thyroid volume as the dependent variable and concentrations of the 7 most toxic PCDD congeners (TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD) as independent variables, cross-tabulation for samples >LOD reduced the N to an insufficient 25. If we omitted the two HxCDD congeners (1,2,3,4,7,8-HxCDD and 1,2,3,7,8,9-HxCDD) with relatively low concentrations, the study population increased to 62 individuals. Model A (supplemental Material, Table S4) shows that with respect to thyroid volume reduction, TCDD was the most potent congener. With FT4 as the endpoint of interest, multiple regression eliminated four PCDF congeners when they were combined with TCDD (Model D in Supplemental Material, Table S4). However multiple regression did not confirm the role of TCDD with FT4 as the dependent variable and PCDD congeners as the independent variable (Model B in Supplemental Material, Table S4) and with thyroid volume as the dependent variable and PCDF congeners as the independent variable (Model C in Supplemental Material, Table S4).

Assessment of REPs for PCDDs, PCDFs and DL-PCBs. Data in Table 1 show that PCDDs were associated with a decrease in both the thyroid volume and the FT4 level. The association between thyroid volume and dioxins decreased with increasing number of chlorine substitutes in the compound except 1,2,3,7,8,9-HxCDD. The PCDFs were associated with a decrease in thyroid volume in a similar way except two compounds, the 1,2,3,4,7,8-HxCDF and OCDF, while with respect to FT4 was observed a mixed response: With 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, 1,2,3,4 6,7,8-HpCDF and OCDF there was a negative association and with 1,2,3,7,8-PeCDF and the 3 listed HxDF congeners a positive association. The DL-PCBs were related to an increase in the thyroid volume and FT4 serum level, except the non-ortho-substituted congener

PCB 81 for both thyroid volume and FT4 and the mono-ortho-substituted congener PCB 105 for FT4. From all congeners, TCDD was most strongly associated with a decrease of thyroid volume and FT4 level. NDL-PCBs (Supplemental Material, Table S5) were associated with slight changes compared to TCDD appearing as increases in most abundant PCB congeners. To comply with the assumption that congeners have a similar mode of action (US EPA 2000), we calculated the REPs only for those acting in the same direction as the index chemical. Thus congeners associated with an increase of thyroid volume or FT4 level were not taken into consideration.

To assess the effect of confounding by other DLC congeners identified in the exposure mixture on regression coefficients, we computed BMCs for thyroid volume decrease related to serum concentration of individual congeners and entered various combinations of congener confounders, while sex and age were always included into the confounder list (confounders 1 and 2). We set both p_0 and benchmark response (BMR)= 0.1 which translates to an increase in risk of 200% (Crump 1995). Based on the Akaike information criterion, the following two regression models were used $f(t) = a_1 + a_2 t$, or $f(t) = a_1 + a_2 t^2$.

We have shown (see Supplemental Material, Table S1) that BMC and benchmark concentration lower confidence limit (BMCL) for TCDD are slightly influenced by the presence of other congeners in the exposure mixture (confounders 3-6). When TCDD was entered as a confounder in combination with other congeners, e.g. with the second most potent congener 1,2,3,7,8-PeCDD, we obtained similar results. Neither adjustment for PCB congeners affected the BMC and BMCL value of TCDD. Therefore we present in Table 1 REPs that were derived after adjustment for sex and age only.

Table 1 contains the REPs calculated as relation of the individual congener slope β_i, BMC_i or $BMCL_i$ to the slope β_{TCDD}, BMC_{TCDD} or $BMCL_{TCDD}$ of the index chemical, respectively.

REPs calculated using the regression slope, BMC, and BMCL data correlated strongly between themselves (all r values >0.903 and $p<0.0001$). Moreover a strong correlation was observed between the REPs calculated from the largely independent thyroid volume and FT4 data. The Spearman correlations were: For REPs derived from thyroid volume and FT4 data using the β_i/β_{TCDD} ($r=0.81$ and $p=0.015$), BMC ($r=0.786$ and $p=0.021$) and BMCL ($r=0.857$ and $p=0.007$) approaches.

The regression slope derived REP data, both for thyroid volume and FT4 level (β_i/β_{TCDD} column in Table 1) correlated significantly with the WHO TEF values (van den Berg et al. 2006) (Spearman $r=0.693$, $p=0.009$ and $r=0.616$, $p=0.033$, respectively). These data are depicted in Figure 1. The best fit can be described as: log REP= 0.566 logTEF -0.229 and logREP= 0.363 logTEF -0.399, for thyroid volume and FT4, respectively. According to our estimates the potencies of congeners above the central axis are greater than the TEFs and vice versa. The BMC and BMCL derived REP data correlated less significantly with the WHO TEF values (data not shown).

In order to view our REPs in a broader context we have included in Table 1 the minimum, maximum and median values published for *in vivo* REPs in the REP 2004 Database (Table 8) (Haws et al. 2006). Our REPs for all PCDD congeners studied and thyroid volume outcome (note that data on 1,2,3,6,7,8-HxCDD are not included in Haws et al. (2006), irrespective of the method of derivation, are between the maximum and minimum values estimated by other researchers, except for OCDD. Our REPs for 1,2,3,4,7,8-HxCDD and 1,2,3,7,8,9-HxCDD,

where FT4 is the outcome, were higher than the published maximum estimates (Haws et al. 2006). Of the 3 REP values for 1,2,3,4,6,7,8-HpCDD, the one obtained as the β_i/β_{TCDD} ratio is smaller than the published maximum estimate (0.029 *versus* 0.035) (Haws et al. 2006). For PCDF congeners associated with thyroid volume, the REPs were close to the maximum values determined by other investigators, except 2,3,4,7,8-PeCDF which still is higher than the minimum reported value of 0.0065 (Haws et al. 2006). For PCDFs and FT4 as an outcome, we calculated REPs for 4 congeners, and of them, values for 2,3,7,8-TCDF and 1,2,3,4,6,7,8-HpCDF were unavailable for comparison, the 2,3,4,7,8-PeCDF fits well between the range published (Haws et al. 2006), while OCDF behaves like an outlier with regard to TEFs.

When analyzing the relative magnitude of thyroid effects of PCB congeners we took into consideration both DL- and NDL-PCB congeners. In Figure 2 we plotted β values for PCB congeners for thyroid volume against those for FT4 serum level shown in Table 1 and Supplemental Material, Table S5. The three non-ortho-substituted PCBs (congeners 81 TEF 0.0003, 126 TEF 0.1 and 169 TEF 0.03) are close to the left end of the line of best fit. PCB 81 is even in the left lower quadrant, the location of TCDD and most dioxins. In this quadrant are also two NDL-PCB congeners, 28 and 52, though both congeners were subject to a high proportion of samples with values below the LOD (40.6% and 11.9%, respectively). PCB 126 and PCB 169 are in the left lower corner of the right upper quadrant with both FT4 and thyroid volume β plus values, together with PCB 118 (TEF 0.00003). The mono-ortho-substituted PCBs, congeners 105, 156, 167, 189, 157, 123 and 114 (TEFs 0.00003) are distributed along the line of best fit ($y = 0.4575x - 0.0026$; $R^2 = 0.8022$). When we added to PCB βs the TCDD β (coordinates -1.101 for thyroid volume and -0.508 for FT4) we obtained the equation $y = 0.4592x - 0.0026$; $R^2 = 0.9988$. The slopes of the two equations were not statistically different, meaning that the line

approximating the PCB section is meeting the distant TCDD point. This analysis suggests continuity between a dioxin-like and a non dioxin-like effect. This is in conformance with the four orders of magnitude difference between TEFs for TCDD and most DL-PCBs.

Discussion

Although some potential environmental hazards involve significant exposure to only a single compound, most instances of environmental contamination involve concurrent or sequential exposures to a mixture, which may induce similar or dissimilar effects over exposure periods ranging from short-term to lifelong (US EPA 2000). Interest in the potential effect of chemical mixtures has increased significantly in the last decade (European Commission 2010; IPCS 2009; Kortenkamp et al. 2007). In this context, study tools such as the Relative Potency Factor (RPF) method have been developed. This approach uses empirically derived scaling factors based on toxicity studies of the effect in combination with exposure conditions of interest in the assessment (US EPA 2000) and is the backbone of our study. The TEF method is a special case of the RPF method (US EPA 2000), and deals with the mixture toxicity of DLCs. The DLCs may serve as a prototype example of mixture toxicity (van den Berg et al. 2006). Relevant studies with DLCs were reviewed extensively by Haws and co-workers within the framework of the TEF concept (Haws et al. 2006). One of the aims of the present study was to place our results in this vast body of scientific knowledge. As far as we know, the current investigation is the first human *in vivo* analysis of REPs of individual mixture components after an exposure to DLCs.

Our study has several unique methodological aspects. First, we obtained six REP values for most congeners evaluated which were derived from the BMC, BMCL, and regression slope approach

for two endpoints, thyroid volume and FT4 serum concentration. The results of the three approaches are so tightly interrelated that any of them can be preferred. A second aspect is that the potential effects of mixture components need to be accounted for. Multivariable regression analysis though has shown that the contribution of confounding congeners to the final outcome was negligible. Therefore, we did not take the confounding congeners, except age and sex, into consideration when calculating REPs. Proceeding in such a way is supported by differences in congener specific mechanisms of action leading to their independent action.

With regard to our study design, several issues have to be considered. First is the selection of endpoints for exposure-effect analysis. We have chosen two thyroid biomarkers, FT4 serum level and thyroid volume, because thyroid pathology in animals and humans exposed to DLCs is most prominent from all other specific toxicological and biological non-cancer health effects (Boas et al. 2009; Crofton et al. 2005; Zoeller et al. 2002; Zoeller 2007; Zoeller 2010, Langer et al. 2010; Rádiková et al. 2008). A decrease of thyroid hormone T4 was recently suggested as a prospective biomarker for generating a new human TEF scheme for DL-PCBs, and circulating T4 decrease is the only consistent biomarker for both DL- and NDL-PCBs. (Yang et al. 2010). This is important as non-coplanar PCBs elicit a diverse spectrum of non-Ah-receptor-mediated toxic responses in humans and animals (Yang et al. 2010). In agreement with this, our results (Figure 2) demonstrate the association between the two thyroid endpoints using simultaneously both DL- and NDL-PCB data. Another issue to be taken into consideration is dose additivity assumed by the TEF method. In a short term study with thyroid hormone disrupting chemicals in rats, depending on dose, both dose additivity and synergism were observed (Crofton et al. 2005); however it can be questioned whether this applies to our long term, low dose, human exposure scenario.

The other thyroid biomarker evaluated in the present study is thyroid size. Estimation of thyroid volume is generally considered to be important in several pathologic situations such as iodine deficiency goiter, thyroiditis, and multinodular goiter (Hegedüs 1990). Regression analysis suggested that serum TSH, serum FT4, sex, age, smoking, and body mass index each played a small, but significant, role for variation in thyroid volume (Hansen et al. 2004). We have been the only group that extensively exploited this biomarker in studying PCB effects in humans (Langer 2010; Rádiková et al. 2008) and the present choice was a logical continuation to our previous studies.

A significant finding in the present study is that the exposure to the index chemical, TCDD, and most DLCs was associated with a decrease in both thyroid volume and FT4 serum concentrations. The FT4 shift is consistent with an observation in a community exposed to dioxin-like congeners (Bloom et al. 2006). On the other hand, exposure to PCBs had a slightly varying association, which was much smaller in magnitude. Regression coefficients for PCBs *versus* thyroid volume and FT4 ranged within 1.65 to –5.7 % and 1.77 to -4.33 %, respectively, from those for TCDD. The parameter increases for the most abundant NDL-PCBs agree with our results with FT4 and thyroid volume (Langer et al. 2010; Rádiková et al. 2008) and with FT4 in anglers (Bloom et al 2009).

A second important issue is the mode of action of the index chemical and of the congeners studied. In REP studies similarity of the mode of action justifies the inclusion of a compound in the TEF concept for DLCs. The inclusion criteria include a structural relationship to TCDD, binding to the Ah receptor, an Ah receptor–mediated biological or toxic responses, and persistence and accumulation in the food-chain (van den Berg et al. 2006). However at present,

there is no published evidence that long-term morphological changes of the thyroid gland and hormonal shifts chosen as endpoints in this study, is an exclusively Ah receptor–mediated process. Earlier, we described a biphasic association between serum concentration of a mixture of PCBs and FT4 (i.e. negative one in the category of PCBs level <530 ng/g *versus* positive one in the category of 531–25 000 ng/g) (Langer et al. 2007c) which makes even more difficult an assignment of a mode of action in humans exposed to complex environmental mixtures of DLCs and NDL-PCBs. In addition, there is no agreement on presence of possible effects of DLCs on thyroid function at environmental exposure levels (Johnson et al. 2001; Pavuk et al. 2003).

In the present study, the REPs calculated via two different approaches, one based on thyroid morphology and the other on thyroid hormonal endpoint, showed consistent results. In spite of different designs among REP studies published, and a rather unique scenario of our study, most of our REPs calculated, especially those for dioxins and thyroid volume, fit well within the ranges of published REPs (Haws et al. 2006) (See table 1). In plots (Figure 1) of log REPs for thyroid volume (Figure 1A) or FT4 (Figure 1B) *versus* log TEFs, however the best fit is markedly shifted in the direction of our REPs. This is more pronounced for FT4, which may be interpreted as a greater sensitivity of this endpoint compared to thyroid volume or to endpoints leading to the assigned TEF values.

The strengths of our study are that it is based on changes of two types of human thyroid data with an apparently completely different pathogenesis, but whose results largely agree. An additional strength is that we used actual serum concentrations of compounds which reliably reflect systemic body burden, rather than data on daily intake. The weakness is that while exposure scenario under laboratory conditions takes into account one single chemical, under

field conditions we are facing exposure to a mixture of chemicals with different potencies and likely different modes of action. Further, single time exposure data does not necessarily reflect the whole exposure history of each subject. In addition, the prevalence of concentrations below the limit of detection for some compounds was high, and this likely limited our statistical precision. In spite of the shortcomings of a study of this type, REPs determined in the current study should be taken into consideration when updating the present TEFs with regard to long-term and low-dose exposure of humans as opposed to relatively short-term animal studies.

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Table 1. The calculated relative effect potencies (REPs) and source data from which they have been calculated -

		Thyroid volume				FT4				TEF[a]	REP2004Database[b]		
	n	β[c]	REP[d] as β_i/β_{TCDD}	REP[d] as BMCL	REP[d] as BMC	β[c]	REP[d] as β_i/β_{TCDD}	REP[d] as BMCL	REP[d] as BMC		Minimum	Median	Maximum
PCDDs													
2378-TCDD	70	-1.101	1	1	1	-0.508	1	1	1	1			
12378-PeCDD	132	-0.45	0.432	0.143	0.325	-0.24	0.471	0.907	0.847	1	0.044	0.4	1.5
123478-HxCDD	81	-0.283	0.257	0.237	0.332	-0.409	0.805	1.413	0.981	0.1	0.0076	0.059	0.35
123678-HxCDD	286	-0.091	0.082	0.049	0.085	-0.064	0.126	0.238	0.256	0.1	_	_	_
123789-HxCDD	76	0.146				-0.245	0.482	1.603	0.853	0.1	0.029	0.029	0.029
1234678-HpCDD	316	-0.009	0.008	0.014	0.011	-0.015	0.029	0.065	0.068	0.01	0.001	0.01	0.035
OCDD	319	-0.003	0.003	0.002	0.001	0.002				0.0003	0.00025	0.00025	0.00025
PCDFs													
2378-TCDF	43	-0.912	0.828	0.629	0.635	-0.051	0.1	0.685	0.128	0.1	_	_	_
12378-PeCDF	13	-0.382	0.347			0.657				0.03	0.0027	0.022	0.95
23478-PeCDF	314	-0.019	0.016	0.011	0.016	-0.01	0.02	0.02	0.03	0.3	0.0065	0.2	3.7
123478-HxCDF	311	0.023				0.043				0.1	0.014	0.05	0.16
123678-HxCDF	312	-0.161	0.146	0.067	0.091	0.012				0.1	0.0031	0.081	0.16
234678-HxCDF	51	-0.86	0.78	0.257	0.322	1.084				0.1	0.015	0.018	0.1
1234678-HpCDF	314	-0.059	0.054	0.083	0.132	-0.027	0.053	0.194	0.083	0.01	_	_	_
OCDF	80	0.127				-0.19	0.373	0.367	0.136	0.0003	0.000004	0.000077	0.0016
DL-PCBs													
PCB 81	234	-0.0111	0.01	0.011	0.025	-0.009	0.017	0.041	0.05	0.0003	_	_	_
PCB 126	319	0.0009				0.000040				0.1	0.000067	0.1	0.86
PCB 169	320	0.0034				0.0022				0.03	0.0000018	0.019	0.74
PCB 105	276	0.0096				-0.0009	0.0019	0.015	0.017	0.00003	0.00000047	0.000042	0.0022
PCB 114	315	0.063				0.0213				0.00003	0.0002	0.00034	0.00048
PCB 118	301	0.0032				0.0005				0.00003	0.00000042	0.00002	0.0023
PCB 123	276	0.033				0.022				0.00003	0.000034	0.000044	0.000055
PCB 156	315	0.0075				0.0022				0.00003	0.0000021	0.000055	0.42
PCB 157	315	0.0291				0.0087				0.00003	0.000420	0.0011	0.0017
PCB 167	315	0.0192				0.0027				0.00003	_	_	_
PCB 189	315	0.0265				0.0117				0.00003	0.000037	0.000055	0.00018

Abbreviations: PCDDs, polychlorinated dibenzo-p-dioxins; PCDFs, polychlorinated dibenzofurans; DL-PCBs, dioxin-like polychlorinated biphenyls

[a] van den Berg et al. 2006 [b] Haws et al., 2006 [c] The slopes were obtained by regression.

[d] The REPs (relative effect potencies) were calculated as the ratios of the BMCs (benchmark concentrations) and BMCLs (benchmark concentration lower confidence limits) or β (regression slopes) of individual congeners to those of the index chemical TCDD. The slopes and benchmark values were adjusted to gender and age.

Figure 1. Relationship between REPs calculated for individual congeners of the exposing DL mixture and the TEFs published (van den Berg et al. 2006). The line is best fit between the logarithms of the two variables. A: Thyroid volume data. B: Serum FT4 data.

Figure 2. A plot of regression coefficients β (Table 1, column β and Supplemental Material, Table S5) derived for thyroid volume *versus* PCB congeners concentration (vertical axis) against those derived for FT4 serum concentration *versus* PCB congeners concentration (horizontal axis).

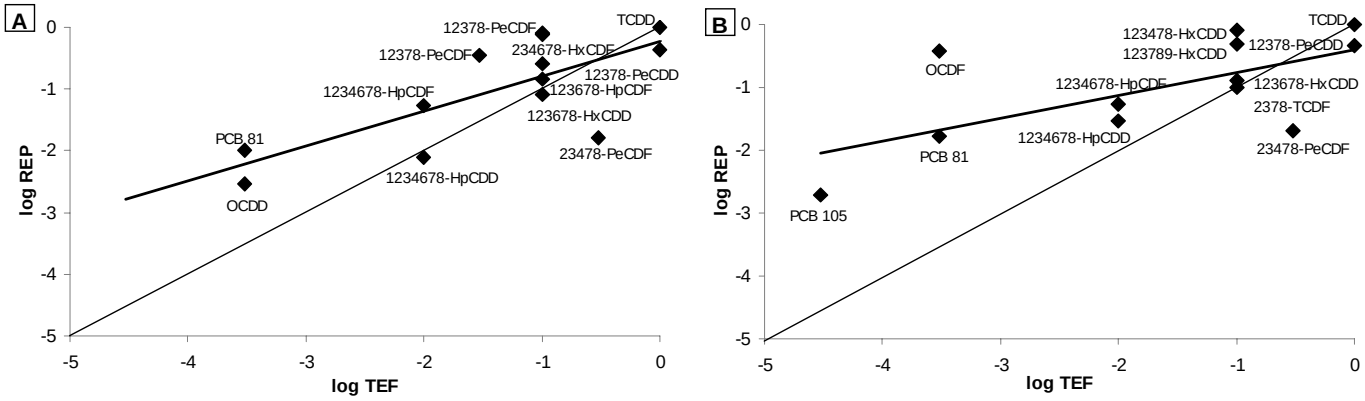
A
log REP
log TEF
0
-1
-2
-3
-4
-5
-5
-4
-3
-2
-1
0
TCDD
12378-PeCDF
234678-HxCDF
12378-PeCDF
12378-PeCDD
1234678-HpCDF
123678-HpCDF
123678-HxCDD
PCB 81
23478-PeCDF
1234678-HpCDD
OCDD
B
log REP
log TEF
TCDD
123478-HxCDD
12378-PeCDD
123789-HxCDD
OCDF
1234678-HpCDF
123678-HxCDD
2378-TCDF
1234678-HpCDD
23478-PeCDF
PCB 81
PCB 105

Figure 1

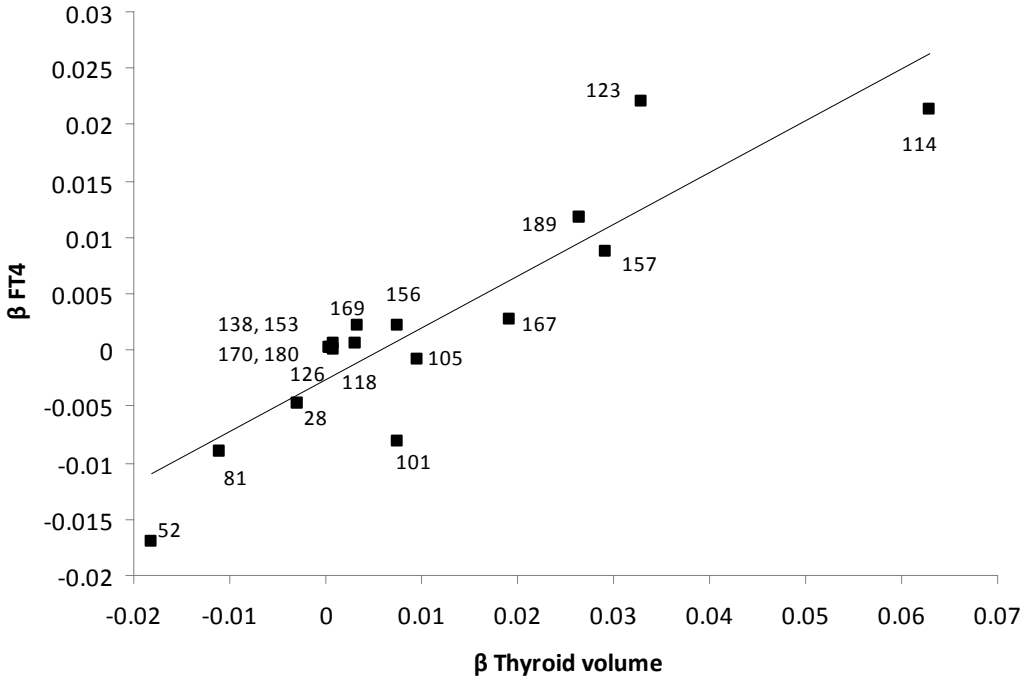
0.03
0.025
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
β FT4
-0.02
-0.01
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
β Thyroid volume
123
114
189
157
156
169
138, 153
167
170, 180
105
126
118
28
101
81
52

Figure 2